INFECTION AND IMMUNITY, Feb. 2004, p. 916–922 Vol. 72, No. 2
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.2.916–922.2004

In Vivo Phase Variation and Serologic Response to Lipooligosaccharide of *Campylobacter jejuni* in Experimental Human Infection

Martina M. Prendergast,[1] David R. Tribble,[2] Shahida Baqar,[2] Daniel A. Scott,[2] John A. Ferris,[1] Richard I. Walker,[3]† and Anthony P. Moran[1]*

Department of Microbiology, National University of Ireland, Galway, Ireland,[1] and Naval Medical Research Center, Silver Spring,[2] and Antex Biologics, Gaithersburg,[3] Maryland

Received 8 April 2003/Returned for modification 23 May 2003/Accepted 22 October 2003

Some *Campylobacter jejuni* strains which exhibit mimicry of gangliosides in their lipooligosaccharides (LOSs) are associated with development of Guillain-Barré syndrome, which complicates the selection of a suitable *C. jejuni* strain in a live-attenuated vaccine. *C. jejuni* 81-176 is the most well characterized strain available, but structurally, LOS of *C. jejuni* 81-176 exhibits mimicry of predominantly GM_2 and GM_3 gangliosides. We compared the antiganglioside human serologic responses of 22 volunteers post-oral vaccination (two-dose series, 14 days apart) with a killed whole-cell *C. jejuni* vaccine, those of volunteers (22 following initial challenge and 5 upon rechallenge) experimentally infected with the homologous *C. jejuni* vaccine strain 81-176, and those of 12 volunteers used as controls (placebo recipients). All volunteers were evaluated using thin-layer chromatography immuno-overlay and a panel of nine gangliosides at days 0, 21, and 28 either postvaccination or postinoculation. Antiganglioside antibodies were identified at baseline in 6 of the 61 volunteers (9.8%). There were no antiganglioside antibodies observed following vaccination or experimental infection rechallenge. Evidence of seroconversion was observed in 2 of 22 (9.1%) in the initial infection challenge group, comparable to 1 of 12 (8.3%) in the placebo recipients. Additional testing of seven selected volunteers in the initial challenge group at days 0, 3, 7, 10, 21, 28, and 60 showed that when antiganglioside antibodies occurred (mostly anti-GM_1 and -GM_2), responses were weak and transient. Furthermore, evidence from serologic probing of LOSs of isolates recovered from stools of six volunteers indicated that the isolates had undergone antigenic phase variation in ganglioside mimicry during passage in vivo. Collectively, with the exception of one volunteer with anti-GM_2 antibodies at day 60, the results show an absence of persistent antiganglioside antibodies after experimental infection with *C. jejuni* or following administration of a killed *C. jejuni* whole-cell oral vaccine, although LOS phase variation occurred.

Campylobacter jejuni is the most commonly reported bacterial cause of human gastroenteritis worldwide (6) and is a major antecedent of traveler's diarrhea (25). Due to the lack of a suitable animal model of virulence, the pathophysiology of *C. jejuni* infection is poorly understood, which in turn has delayed vaccine development efforts. In addition, the link between *C. jejuni* infection and development of reactive arthritis, as well as that between *C. jejuni* infection and the postinfectious neurological complications Guillain-Barré syndrome (GBS) and Miller Fisher syndrome, further increases the public health and economic burdens of *C. jejuni*-associated disease (2). Importantly, *C. jejuni* is antigenically complex, as demonstrated by the presence of more than 70 heat-stable (HS) serotypes (14).

Lipopolysaccharide and lipooligosaccharides (LOSs) are HS antigens on the surface of gram-negative bacteria, and many *C. jejuni* HS serotypes have been shown to bear LOS structures that mimic human gangliosides, components of nerve tissue (21). It is thought that this molecular mimicry is responsible for the induction of antiganglioside antibodies, which are considered to play a role in GBS and/or Miller Fisher syndrome development (15, 21). Also, it is now evident that *C. jejuni* LOS undergoes high-frequency phase variation (7, 8, 10, 12, 13), and one strain of *C. jejuni* can produce a repertoire of LOS molecules. This complicates the selection of a suitable *C. jejuni* strain as a candidate for a live-attenuated vaccine. Thus, the development of a vaccine to control rising *C. jejuni* incidences must take into account the phenomenon of molecular mimicry of gangliosides by *C. jejuni* LOS, phase variation, and the lack of a conserved protective antigen.

In the present study, using thin-layer chromatography (TLC) with immunostaining, we serologically characterized LOS from *C. jejuni* 81-176, a reference strain currently being developed as a killed whole-cell vaccine, using a panel of ganglioside-specific and ligand-binding reagents. Also, a unique opportunity to define the antiganglioside human serologic responses resulted upon completion of a volunteer experimental oral infection study with the *C. jejuni* 81-176 reference strain (D. Tribble, S. Baqar, M. Oplinger, D. Scott, D. Rollins, S. Walz, E. Burg, A. Moran, and A. Bourgeois, Abstr. 10th Int. Congr. Immunol., abstr., p. 424, 1998). Our aim was to investigate the potential of the strain to trigger an antiganglioside response and, thus, contribute to neurological symptoms. We monitored the serologic response to gangliosides at three time points (0,

* Corresponding author. Mailing address: Department of Microbiology, National University of Ireland, Galway, University Rd., Galway, Ireland. Phone: 353-91-524411, ext. 3163. Fax: 353-91-525700. E-mail: anthony.moran@nuigalway.ie.

† Present address: Division of Bacterial, Parasitic and Allergenic Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD 20851-1448.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2004	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
In Vivo Phase Variation and Serologic Response to Lipooligosaccharide of Campylobacter Jejuni in Experimental Human Infection	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Naval Submarine Medical Research Laboratory Naval Submarine Base New London Box 900 Bldg 148, Trout Avenue Groton, CT 06349-5900**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**7**	

21, and 28 days) during the trial. Furthermore, we serologically characterized the LOS from six *C. jejuni* isolates recovered from volunteers after passage and compared it to the LOS from the reference strain.

MATERIALS AND METHODS

Administration of CWC vaccine. *C. jejuni* 81-176 (Penner serotype HS:23/36; Lior serogroup 5) was used following inactivation in the *Campylobacter* whole-cell (CWC) vaccine (25). The strain was previously isolated from the feces of a 9-year-old girl with diarrhea during a milk-borne outbreak in Minnesota (11). The strain has subsequently been studied in an experimental infection study of volunteers at the Center for Vaccine Development, University of Maryland (1). The inactivated whole-cell vaccine was produced using good manufacturing practices and good laboratory practices at the Walter Reed Army Institute of Research Forest Glen Annex facility through a Cooperative Research and Development Agreement with Antex Biologics, Gaithersburg, Md. The vaccine dose was delivered as a suspension in a bicarbonate buffer solution (4.0 g of USP-grade sodium bicarbonate dissolved in 150 ml of sterile water) containing 2.5×10^9 CFU coadministered with LT(R192G) as mucosal adjuvant, a genetically modified heat-labile toxin of enterotoxigenic *Escherichia coli*. LT(R192G) was constructed as described by Dickinson and Clements (3) with purification using agarose affinity chromatography, and the Swiss Serum Vaccine Institute, Bern, Switzerland, under contract from the Naval Medical Research Center, produced the good-manufacturing-practices lot. The adjuvant was added as a separate suspension in a total volume of 5 ml (25 μg) with the CWC vaccine just prior to ingestion. The placebo consisted of only bicarbonate buffer. The vaccine was delivered as a two-dose regimen at a 14-day interval. The vaccination procedure was as follows: 90-min fast, delivery of assigned treatment in buffer drink (ingested as a combined solution), verification of full vaccination, and postdose 90-min fast. All vaccinations were done at the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, in Frederick, Md.

Following informed consent, civilian and military volunteers underwent evaluations to determine eligibility, including medical history, physical examination, and screening laboratory and preenrollment testing to assess their understanding of the study. Volunteers were excluded from enrollment due to chronic gastrointestinal disorders, personal or family history of an inflammatory arthritis, autoimmune or neurologic disease, pregnancy, HLA B27 positivity, human immunodeficiency virus seropositivity, or any disease or condition as determined by a study physician that would place the subject at increased risk for adverse effects. Additional exclusions not related to volunteer safety included previous vaccination with experimental *Campylobacter* vaccines, traveler's diarrhea in the past year, and history of microbiologically confirmed *Campylobacter* infection or serologic evidence of infection (defined as *Campylobacter*-specific antiglycine extract immunoglobulin A [IgA]; optical density [OD] at 650 nm > 0.5 at 1:1,000 dilution). The same eligibility criteria were used for all volunteers in either the vaccination or infection groups.

Administration of *C. jejuni* 81-176 reference strain to volunteers. A *C. jejuni* strain 81-176 experimental infection challenge study was undertaken to assess preliminary efficacy of the CWC vaccine. The organism, prepared from the master seed stock stored at −85°C in glycerol thioglycolate soy broth, was administered at an inoculum of 10^9 CFU. *C. jejuni* confirmation used standard microbiologic methods, and viable colony counts correlated with OD readings. Inocula were prepared from individual confirmed colonies. These colonies were suspended in thioglycolate soy broth and then plated onto *Brucella* agar (Difco, Detroit, Mich.) overnight at 42°C in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) for confluent growth. Growth was harvested in 5 ml of phosphate-buffered saline (PBS) and adjusted to a target OD with PBS for standardizing a subsequent infectious dose. Pre- and postdose colony counts were performed. The appropriate inoculum (10^9 CFU) was suspended in a solution containing 2 g of sodium bicarbonate in 150 ml of sterile water and then ingested by the volunteer. Volunteers were admitted to an inpatient research ward for daily monitoring. All volunteers were treated with antibiotics (no later than 1 week after inoculation) and had documented resolution of symptoms and eradication of infection prior to discharge.

Blood and stool specimen collection schedule. For specimen collection, blood was collected throughout the course of the study to measure the immune response to vaccination or experimental infection based on group assignment. Comparable time points available for study for all groups were selected for measurement of antiganglioside antibody determination. Blood samples were processed to remove mononuclear cells for immunologic testing in a related study (Tribble et al., Abstr. 10th Int. Congr. Immunol.), and to avoid additional blood collection, plasma samples were used for serologic testing. Importantly, in other studies, we did not find a difference in antibody levels between plasma and serum samples (S. Baqar and A. P. Moran, unpublished data). Plasma samples were stored at −70°C at the time of initial processing and shipped on dry ice for immediate analysis. Study days 0 (baseline prior to intervention), 21, and 28 were selected for comparison among 61 volunteers ($n = 183$ samples). More-frequent blood specimen time points were available following experimental infection. Subsequent to the initial screening, four volunteers who showed antiganglioside antibodies at one or more of these test time points and three others who did not exhibit antibodies at these time points were selected. Additional testing was performed on plasma samples from these seven volunteers taken at 0, 3, 7, 10, 21, 28, and 60 days ($n = 49$ samples). All stool specimens obtained during inpatient observation were cultured for the presence of *C. jejuni*.

TABLE 1. Antibodies used in characterization of *C. jejuni* LOS preparations

Antibody specificity	Host	Antibody class	Clonality	Source
Anti-GM_1	Rabbit	IgG	Polyclonal	MI[a]
Anti-asialo-GM_1	Rabbit	IgG	Polyclonal	MI
Anti-GM_2	Rabbit	IgG	Polyclonal	MI
Anti-asialo-GM_2	Rabbit	IgG	Polyclonal	MI
Anti-GD_2	Mouse	IgG	Monoclonal	MI
Anti-GD_3	Mouse	IgG	Monoclonal	MI
Anti-disialosyl	Human	IgM	Monoclonal	INS[b]
Anti-*C. jejuni* HS:10 serostrain	Rabbit	IgG	Polyclonal	NMRC[c]

[a] Antibody was obtained from Matreya Inc., Pleasant Gap, Pa.
[b] Antibody was provided by H. Willison, University Department of Neurology, Institute of Neurological Sciences, Southern General Hospital, Glasgow, Scotland.
[c] Antibody was provided by P. Guerry, Naval Medical Research Center.

Growth conditions of *C. jejuni* 81-176. For analysis, the reference strain *C. jejuni* 81-176 was cultured separately on blood agar (Columbia agar base [CBA; Oxoid Ltd., London, England], or *Brucella* agar base with 10% (vol/vol) unlysed horse blood (BBA) or on brain heart infusion (BHI) agar (Oxoid) under microaerobic conditions (16). Isolates were recovered from the stools of six volunteers, and bacterial identification was accomplished by established procedures (26). Recovered isolates were stored in glycerol solution at −70°C. Isolates were cultured from the frozen stocks onto the three media described above and grown under the same conditions.

Extraction of LOSs. Bacterial biomass was harvested and bulk extraction of *C. jejuni* 81-176 LOS was performed by the hot phenol-water extraction procedure (16). For the *C. jejuni* isolates recovered from experimentally infected volunteers, the LOSs were extracted by a mini-phenol-water extraction procedure (19).

Electrophoresis. The purified LOS preparations from the *C. jejuni* reference strain and from the six recovered isolates, each grown in the three culture media (CBA, BBA, and BHI), were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a stacking gel of 5% (wt/vol) acrylamide and a separation gel of 15% (wt/vol) acrylamide containing 3.2 M urea (BDH Laboratory Supplies, Poole, England), followed by silver staining (20).

Sialic acid determination. Concentrations of sialic acid (Neu5Ac, *N*-acetylneuraminic acid) were determined in LOSs by the modified Ehrlich reaction assay (4).

TLC and immunostaining. For characterization of the ganglioside mimicry expressed by strains, gangliosides (1-μg aliquots; Sigma Chemical Co., St. Louis, Mo.) and LOS extracts (1 μg or 5-μl aliquots) were analyzed by TLC on precoated silica gel 60 glass plates (Merck, Darmstadt, Germany). Solvent systems consisting of chloroform–methanol–0.22% $CaCl_2 \cdot 2H_2O$ (50:45:10 [vol/vol/vol]) (23), and *n*-propanol–water–25% NH_4OH (60:30:10 [vol/vol/vol]) (24) were used as developers for gangliosides and LOSs, respectively. Separated gangliosides and LOSs were visualized by spraying plates with resorcinol-HCl reagent (24). TLC with immunostaining was performed using the immuno-overlay procedure of Saito et al. (23), as modified by Schwerer et al. (24). LOS preparations were tested for reaction with the antiganglioside antibodies and other antibodies listed in Table 1, with the appropriate peroxidase-conjugated secondary antibody (Sigma) diluted 1:500 in 0.3% gelatin–PBS solution (19). Immunoreactants were visualized by use of a horseradish-peroxidase development system (Bio-Rad Laboratories, Hercules, Calif.). Alternatively, to assay the ganglioside-reactivity of human plasma samples, lanes were overlaid with sera diluted 1:100 in gelatin-

PBS. This procedure was chosen, rather than enzyme-linked immunosorbent assay (ELISA), since the former has been applied and validated in previous serologic studies on *C. jejuni* LOS and antiganglioside antibodies (17–20, 24) and optimal presentation of the relevant ganglioside epitopes for detection by anti-*C. jejuni* antibodies occurs in TLC immuno-overlay (A. P. Moran, unpublished data). Positive and negative control experiments for antibody binding were performed whereby serum from a patient with a high titer of anti-GM_1 IgG antibodies was used (a generous gift from B. Schwerer, Vienna, Austria), or normal human serum without antiganglioside antibodies was used instead of plasma from volunteers. Confidence intervals for antiganglioside antibody responses were generated using a normal approximation to the binomial distribution. Binding studies with cholera toxin (CT)-peroxidase conjugate (1 μg/ml; Sigma) and peanut agglutinin-peroxidase conjugate (20 μg/ml; Kem-En-Tec, Copenhagen, Denmark) were performed under the same conditions as those described for immunostaining (19).

RESULTS

Electrophoretic characterization of LOSs from the reference strain and recovered isolates. All the hot phenol-water-extracted LOSs from the *C. jejuni* 81-176 reference strain, independent of growth medium, had the same electrophoretic profile of a low-M_r LOS (data not shown), identical to that observed previously (10). Furthermore, LOSs extracted from *C. jejuni* 81-176 using the mini-phenol-water procedure (19) gave a profile identical to those obtained using LOSs extracted by the lengthier phenol-water extraction procedure (16). We have previously validated the mini-phenol-water technique and demonstrated that it is a suitable extraction procedure for LOS and yields material sufficiently pure for subsequent analysis by TLC immunostaining (19). As with the reference strain, the LOSs from each of the six recovered isolates, grown in all three media (18 LOS preparations [Table 2]), produced low-M_r bands in silver-stained gels but, compared with the reference strain, differences in LOS banding pattern of the isolates were observed similar to those seen previously in phase-variable LOSs (10).

Neu5Ac determination. The LOS of the reference strain that was grown on CBA had the highest concentration of Neu5Ac (119.1 nmol/mg), whereas Neu5Ac was detected at concentrations of 92.1 and 87.4 nmol/mg in the LOSs of the BBA- and BHI-grown strains, respectively. Thus, medium composition influenced the amount of Neu5Ac expressed in the LOSs of *C. jejuni* 81-176 and is reflective of phase variation in ganglioside mimicry by LOS of the reference strain in vitro (10). Thus, in subsequent serologic probing of the recovered isolates for ganglioside mimicry (see below), analysis was performed on each strain grown on these three respective media (Table 2). Nevertheless, in vitro selection of specific types of ganglioside mimicry, not reflective of what may take place in vivo, could occur in the recovered isolates when grown in vitro, and thus isolates were subjected to minimal subculturing before testing. This led to limitations in available amounts of biomass from the recovered isolates for purification of LOSs, and since the requirement of material for serologic characterization was paramount, the Neu5Ac contents of these LOSs were not determined.

Serologic characterization of LOSs from the reference strain and recovered isolates. To determine the ganglioside mimicry occurring in *C. jejuni* LOSs, serologic probing was used since small amounts of purified LOSs were available, particularly from recovered isolates, which were sufficient for

TABLE 2. Reaction of antibodies and ligands with LOSs of *C. jejuni* reference strain 81–176 and six isolates from inoculated volunteers when grown on three media[a]

Antibody or ligand	Positive control	Strength of reaction with LOS of *C. jejuni*[b]:																	
		Reference strain 81–176	05600			05601			06052			06053			06072			06518		
			CBA	BBA	BHI	CBA	BBA	BHI	CBA	BBA	BHI	CBA	BBA	BHI	CBA	BBA	BHI	CBA	BBA	BHI
Antibodies																				
Anti-GM_1	Strain HS:41	–	+++	++	+++	+++	++	+++	+++	++	+++	(+)	+++	++	+++	+++	+++	++	+++	++
Anti-asialo-GM_1	Strain HS:2[c]	–	(+)	++	+++	(+)	++	+++	++	++	+++	(+)	+++	+++	++	+++	+++	++	+++	+++
Anti-asialo-GM_2	Asialo-GM_2	–	(+)	ND	ND	(+)	ND	ND	(+)	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND
Anti-GM_2	GM_2	–	+	++	++	+	++	++	+	+++	++	(+)	+++	++	(+)	++	++	(+)	+++	++
Anti-GD_2	GD_2	–	(+)	ND	ND	(+)	ND	ND	(+)	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND
Anti-disialosyl	Strain PG836	–	+++	ND	ND	++	ND	ND	++	ND	ND	(+)	ND	ND	++	ND	ND	+	ND	ND
HS:10 antiserum	Strain HS:10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ligands[d]																				
CT-HRP	Strain HS:41	–	+	++	+	(+)	(+)	+	(+)	(+)	+	–	–	–	–	–	–	–	–	–
PNA-HRP	Strain HS:2[c]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Culture growth media: CBA, Columbia blood agar; BBA, *Brucella* blood agar; BHI, brain heart infusion agar (no blood). The reference strain, 81–176, was grown on all three media like the other *C. jejuni* isolates.
[b] +++, strong reaction; ++, moderate reaction; +, weak reaction; (+), very weak reaction; –, no reaction; ND, not determined.
[c] Desialylated *C. jejuni* NCTC 11168 LOS was used.
[d] Ligands: CT-HRP, cholera toxin-peroxidase conjugate; PNA-HRP, peanut agglutinin-peroxidase conjugate.

TABLE 3. Seropositive reactions among plasma samples (n = 183) of the 61 volunteers to gangliosides[a]

Volunteer	Group	Day of sampling	Strength of reaction[c] with ganglioside:								
			Asialo-GM_1	GM_1	GD_{1a}	GD_{1b}	GM_2	GD_2	GD_3	GT_{1b}	GQ_{1b}
GBS[b]			++++	++	−	++	(+)	−	−	−	−
1	Vaccine	0	−	−	−	−	+	−	−	−	−
2	Experimental infection (rechallenge)	0	−	+	−	−	(+)	−	−	+	−
3	Placebo	0	+	+	+	+	+	+	+	−	+
4	Placebo	0	−	(+)	−	−	+	−	−	−	−
5	Placebo	28	−	+	−	−	−	−	−	−	−
6	Experimental infection (initial)	21	−	−	−	−	+	−	−	−	−
		28	(+)	−	−	(+)	+	−	−	−	−
7	Experimental infection (initial)	0	−	+	−	−	+	−	−	−	−
		28	−	+	−	−	+	−	−	+	−
8	Experimental infection (initial)	0	+	++	+	+	++	+	−	++	−
		21	+	+	+	+	++	−	−	+	−
		28	−	−	−	−	+	(+)	−	(+)	−

[a] Plasma samples (n = 183) were screened using TLC immunostaining to detect antiganglioside antibodies at day of infection (day 0) and postinoculation (days 21 and 28) in all volunteers. Only volunteers with positive serologic reactions after screening are shown.
[b] Control serum containing anti-GM_1 antibodies from a patient with Guillain-Barré syndrome.
[c] ++++, very strong reaction; +++, strong reaction; ++, moderate reaction; +, weak reaction; (+), very weak reaction; −, no reaction.

serologic probing but not complete structural analysis. In addition to analysis of the recovered isolates, the reference strain was examined for comparison. Our previous structural studies have established that *C. jejuni* 81-176 LOS expresses mimicry of predominantly GM_2 and GM_3 gangliosides but also expresses, to a minor extent, mimicry of GD_2 and GD_{1b} gangliosides (10; A. P. Moran et al., unpublished data). As shown in Table 2, tests for reactivity of reference strain LOSs with anti-GM_1 antibodies were negative, consistent with the absence of GM_1 mimicry. Antibodies to gangliosides asialo-GM_1, GM_2, asialo-GM_2, GD_2, and GD_3 and to disialosyl disaccharides did not react with the LOSs but reacted with a panel of control gangliosides and *C. jejuni* LOSs which are known to bear these ganglioside structures. Also, results of testing with CT (a GM_1 ligand) and peanut agglutinin (a Gal→GalNAc ligand) indicated the absence of mimicry of the major gangliosides implicated in GBS development. Compared with the structural data, the lack of reaction of *C. jejuni* 81-176 LOSs with anti-GD_2 antibodies is likely attributable to the low proportion of molecules with this type of mimicry in the LOSs of the reference strain. Although the anti-GM_2 antibodies reacted with control GM_2, the lack of reaction of these antibodies with reference strain LOSs contrasts with the structural findings of predominant expression of GM_2 and GM_3 mimicry. Nevertheless, these antibodies did not react with another control *C. jejuni* LOS bearing GM_2 ganglioside mimicry (serostrain HS:23), identical to the observations with reference strain LOSs. These results reflect the importance of epitope presentation for detection of ganglioside-like epitopes in *C. jejuni* LOSs by antiganglioside antibodies.

Remarkably, as shown in Table 2, LOS from each passaged isolate reacted with a range of antiganglioside antibodies and ganglioside-specific ligands and, thus, bore recognizable ganglioside epitopes. For each isolate, the spectrum of reactions of their LOSs with ganglioside-detecting reagents were independent of growth medium, although sometimes differing in strength. In general, *C. jejuni* LOSs from all these isolates reacted very strongly with anti-GM_1 and -GM_2 antibodies, suggesting the occurrence of such mimicry. However, the lack of binding of CT (a GM_1 ligand) in three of the six isolates did not confirm GM_1 mimicry in these isolates. Due to reaction of LOSs from all the six isolates with antidisialosyl antibodies, a neuroaminobiose unit on a GM_1/GM_2-like OS backbone is proposed. In addition, for some of the isolates weak reactions with anti-GD_2 antibodies were observed. Thus, in contrast to the absence of serologically detectable mimicry by the major gangliosides in the reference strain, after in vivo passage the isolates possess a Neu5Acα2-3Gal or Neu-5Acα2-8-Neu5Aca2-3Gal epitope in the form of GM_2/GD_2/GD_{1b} mimicry. Hence, the *C. jejuni* LOS underwent antigenic phase variation during passage in vivo. Consistent with these findings, a previous in vitro study has shown that *C. jejuni* 81-176 possesses the genetic mechanisms for its LOS to undergo phase variation resulting in not only expression of GM_2 and GM_3 ganglioside-like structures but also GD_{1b} and GD_2 ganglioside mimicry (10).

Testing of human sera for ganglioside reactivity. The serologic response to a panel of gangliosides in plasma samples (n = 183) obtained at the day of intervention (day 0), vaccination or infection, and 21 and 28 days postintervention was studied. At baseline (prior to intervention) 6 of the 61 volunteers, or 9.8% (95% confidence interval [CI], 2.4 to 17%), exhibited a serological response to at least one of the gangliosides in the panel. There were no seroconversions among the 22 volunteers in the CWC vaccine group or the 5 volunteers following their rechallenge to the experimental infection. All of the 22 volunteers in the experimental infection group became infected, and 17 (77%) became ill, with clinical outcomes ranging from mild to severe diarrhea. Two of the 22 volunteers, 9.1% (95% CI, 0 to 21%), seroconverted to one or more of the gangliosides after their initial infection. This rate was similar to that observed in the placebo recipients, 8.3% (95% CI, 0 to 24%), following no intervention. All volunteers that exhibited a serological response at any time point (day 0, 21, and/or 28) are included in Table 3. In general, the samples that tested positive for the presence of antiganglioside antibodies had only weak responses and were mostly directed against GM_1/asialo-GM_1 and GM_2 gangliosides, although some volunteers exhib-

ited serological responses against a wider variety of gangliosides. One positive sample, that from volunteer 3 taken at day 0, had weak antibody responses to eight of nine gangliosides tested. Similarly, another five of the eight volunteers who tested positive (volunteers 1, 2, 4, 7, and 8) had positive samples at day 0 prior to any intervention. Hence, these responses were not induced by an exposure to either vaccine or infection. Furthermore, the occurrence of an antiganglioside response at the subsequent time points of 21 or 28 days in volunteers 7 and 8 was consistent with the presence of such antibodies on day 0. Nevertheless, for the three remaining plasma samples (volunteer 5, day 28; volunteer 6, days 21 and 28) antiganglioside serological responses (anti-GM_1, -asialo-GM_1, -GD_{1b}, and -GM_2) were observed which were absent on day 0 (one in the placebo group and two from the initial infection group, respectively). Importantly, none of the volunteers developed GBS.

Therefore, to better assess these postinfection observations, four volunteers who showed antiganglioside antibodies at one or more of these test time points (volunteers 2, 6, 7, and 8) and three others who did not exhibit antibodies at these time points (volunteers 9, 10, and 11) were selected for additional testing. Plasma samples from these seven volunteers taken at days 0, 3, 7, 10, 21, 28, and 60 ($n = 49$ samples) were assayed. Table 4, which summarizes the results of this testing, shows that the most frequent antibody responses were observed against GM_2, GM_1, GD_{1a}, GD_{1b}, asialo-GM_1, and GT_{1b}. Volunteers 2, 7, and 8 had weak or moderate responses to GM_1 and GM_2 on day 0, but this response disappeared in volunteer 2 by day 21. In these three volunteers, antibodies to these and other gangliosides were weak and transient or, if persistently present at later time points (e.g., 21 to 60 days), had been previously detectable at day 0, indicating the serological responses were not induced by the infection. Although plasma samples from volunteers 6, 9, 10, and 11 had no antiganglioside antibodies on day 0, a weak transient response to GM_1 and GM_2 gangliosides developed in volunteers 9, 10, and 11, which was absent by day 21. In volunteer 6, although a weak response to GM_2 was observed even at day 60, only a weak transient response to GM_1 occurred. No further responses to the remaining gangliosides of the test panel were observed in volunteers 10 and 11, and the limited responses to other gangliosides in volunteers 6 and 9 were transient. Furthermore, using a procedure for optimal coating of gangliosides on ELISA plates (5), we formulated an antiganglioside ELISA which gave comparable results to those obtained with the TLC-immuno-overlay technique for these seven volunteers (data not shown). Collectively, with the exception of one volunteer (volunteer 6, anti-GM_2 antibodies) the results demonstrate an absence of induction of persistent antiganglioside antibodies after experimental infection with *C. jejuni* 81-176.

TABLE 4. Serologic reactions of plasma samples ($n = 49$) from selected *C. jejuni* experimentally infected volunteers ($n = 7$) at additional time points[a]

Volunteer	Day of sampling	Strength of reaction with ganglioside[b]:							
		Asialo-GM_1	GM_1	GD_{1a}	GD_{1b}	GM_2	GD_2	GD_3	GT_{1b}
2	0	−	+	−	−	(+)	−	−	+
	3	−	+	−	+	+	−	−	+
	7	+	++	+	+	+	−	+	+
	10	+	++	+	+	+	+	+	+
	21	−	−	−	−	−	−	−	−
	28	−	−	−	−	−	−	−	+
	60	−	−	−	−	−	−	−	−
6	0	−	−	−	−	−	−	−	−
	3	+	++	+	+	+	−	−	−
	7	−	−	−	−	−	−	−	−
	10	−	+	−	−	++	−	−	−
	21	−	−	−	−	+	−	−	−
	28	(+)	−	−	(+)	+	−	−	−
	60	−	−	−	−	+	−	−	−
7	0	−	+	−	−	+	−	−	−
	3	−	+	−	−	+		−	−
	7	−	−	−	−	−	−	−	−
	10	−	+	−	−	+	−	−	+
	21	−	−	−	−	−	−	−	−
	28	−	+	−	−	+	−	−	+
	60	−	+	−	−	−	−	−	−
8	0	+	++	+	+	++	+	−	++
	3	+	−	−	−	+	−	−	(+)
	7	(+)	+	(+)	−	++	−	−	+
	10	−	+	−	−	+	−	−	(+)
	21	+	+	+	+	++	−	−	+
	28	−	−	−	−	+	(+)	−	(+)
	60	−	+	−	−	++	(+)	−	(+)
9	0	−	−	−	−	−	−	−	−
	3	−	++	+	+	+	+	−	−
	7	−	−	−	−	−	−	−	−
	10	+	+	(+)	−	+	−	+	−
	21	−	−	−	−	−	−	−	−
	28	−	−	−	−	−	−	−	−
	60	−	−	−	−	−	−	−	−
10	0	−	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−	−
	7	−	+	−	−	+	−	−	−
	10	−	−	−	−	−	−	−	−
	21	−	−	−	−	−	−	−	−
	28	−	−	−	−	−	−	−	−
	60	−	−	−	−	−	−	−	−
11	0	−	−	−	−	−	−	−	−
	3	−	+	−	−	+	−	−	−
	7	−	+	−	−	+	−	−	−
	10	−	−	−	−	+	−	−	−
	21	−	−	−	−	−	−	−	−
	28	−	−	−	−	−	−	−	−
	60	−	−	−	−	−	−	−	−

[a] Plasma samples ($n = 49$) were screened using TLC immunostaining to detect antiganglioside antibodies at the day of infection (day 0) and postinoculation (days 3, 7, 10, 21, 28, and 60) in the seven selected volunteers; four volunteers (2, 6, 7, and 8) had shown antiganglioside antibodies in initial screening at day 0, 21, or 28 (Table 3), and three other volunteers (9, 10, and 11) did not. Additional testing was performed on plasma samples from these seven volunteers taken at days 0, 3, 7, 10, 21, 28, and 60 ($n = 49$ samples).

[b] In addition to testing for reaction with the gangliosides listed, plasma samples were screened for reaction with GQ_{1b} but no reactions were observed. Symbols: ++, moderate reaction; +, weak reaction; (+), very weak reaction; −, no reaction.

DISCUSSION

C. jejuni 81-176 is a very well characterized strain, was originally isolated from the feces of a 9-year-old girl with diarrhea (11), and has been shown to induce diarrhea in human volunteers (1). In the present work, studies were undertaken arising from the completion of a volunteer experimental oral infection study with the *C. jejuni* 81-176 reference strain (Tribble et al., Abstr. 10th Int. Congr. Immunol.).

Initially, LOSs from the *C. jejuni* 81-176 reference strain and six recovered isolates from volunteers were characterized. To determine the ganglioside mimicry occurring in *C. jejuni* LOSs, serologic probing was used since small amounts of purified LOSs were available, particularly from recovered isolates, which were sufficient for serologic probing but not complete structural analysis. Structural studies have established that *C. jejuni* 81-176 LOS expresses mimicry of predominantly GM_2 and GM_3 gangliosides, but also to a minor extent of GD_2 and GD_{1b} gangliosides (10; A. P. Moran et al., unpublished data). Antiganglioside antibodies and ganglioside-specific ligands did not react with the purified LOS of the reference strain, and collectively, the results indicated the absence of mimicry by the major gangliosides implicated in GBS development. Compared with the structural data, the lack of reaction of *C. jejuni* 81-176 LOSs with anti-GD_2 antibodies is likely attributable to the low proportion of molecules with this type of mimicry in the LOSs of the reference strain. Although the anti-GM_2 antibodies reacted with control GM_2 ganglioside, the lack of reaction of these antibodies with reference strain LOSs contrasts with the structural findings of predominant expression of GM_2 and GM_3 mimicry. However, these antibodies did not react with another control *C. jejuni* LOS bearing GM_2 ganglioside mimicry (serostrain HS:23), identical to the observations with reference strain LOSs. These results reflect the importance of epitope presentation for detection of ganglioside-like epitopes in *C. jejuni* LOSs by antiganglioside antibodies and are consistent with previous findings that the core sugars in *C. jejuni* LOS, on which the ganglioside-mimicking structures are carried, can influence epitope recognition (19, 20).

Remarkably, in contrast to the absence of serologically detectable mimicry by the major gangliosides in the reference strain, after in vivo passage the isolates possess a Neu5Acα2-3Gal or Neu-5Acα2-8-Neu5Aca2-3Gal epitope in the form of $GM_2/GD_2/GD_{1b}$ mimicry. Hence, the *C. jejuni* LOS underwent antigenic phase variation during passage in vivo. Importantly, this is the first report of in vivo phase variation by *C. jejuni* LOS in humans. Consistent with these findings, a previous in vitro study has shown that *C. jejuni* 81-176 possesses the genetic mechanisms for its LOS to undergo phase variation, resulting in expression of GM_2 and GM_3 ganglioside-like structures but also in GD_{1b} and GD_2 ganglioside mimicry (10). Furthermore, comparative sequence analysis of the *cgtA* gene (which encodes a phase-variable *N*-acetylgalactoaminyltransferase involved in synthesis of $GM_2/GD_2/GD_{1b}$ structures) from individual colonies of the recovered isolates and the reference strain indicated the occurrence of homopolymeric tracts whose length is consistent with an actively expressed enzyme (10) in all colonies of the recovered isolates examined (A. P. Moran, unpublished results). Also, our comparative electrophoretic analyses of the reference strain and isolates showed differences in LOS banding patterns similar to those observed previously in phase-variable LOSs (10).

Subsequently, the serologic response to a panel of gangliosides in plasma samples ($n = 183$) obtained at the day of intervention (day 0), vaccination or infection, and 21 and 28 days postintervention was assessed with the TLC-immunooverlay technique. Overall, only 12 of 183 (6.6%) of the plasma samples tested from eight volunteers were positive for the presence of antiganglioside antibodies, irrespective of intervention group. Of the subjects testing positive, six volunteers had antibodies on day 0 of the trial, and thus the serologic responses in these volunteers were not induced by the intervention. Evidence of seroconversion was observed in 2 of 22 (9.1%) volunteers in the initial infection challenge group, comparable to 1 of 12 (8.3%) in placebo recipients, following no intervention.

Further testing of seven selected volunteers at additional time points of 0, 3, 7, 10, 21, 28, and 60 days ($n = 49$ samples) showed that when antiganglioside antibodies occurred (mostly anti-GM_1 and -GM_2), responses were weak and transient. Only one volunteer showed a weak response to GM_2 even at day 60, and with the exception of this volunteer, the results showed an absence of persistent antiganglioside antibodies after CWC vaccination or experimental infection with *C. jejuni* 81-176. Furthermore, testing of plasma samples from these seven selected volunteers in an antiganglioside ELISA confirmed these findings. Nevertheless, we cannot exclude the possibility that because of the rare incidence of GBS a much larger group of volunteers would be required for antiganglioside antibodies to be detected. Also, knowledge of the kinetics and the quantity of the antiganglioside response to induce GBS is lacking from the scientific literature, and thus, at present, it is not possible to define what quantity of a response would induce GBS in a susceptible host. Furthermore, although immunogenetic factors may play a role in the pathogenesis of GBS, efforts to demonstrate an association between human leukocyte antigen (HLA) types and the development of *C. jejuni*-associated GBS have been inconclusive (21). Thus, the requirements for an individual to be susceptible to GBS development after *C. jejuni* infection remain to be identified since HLA type has not been consistently correlated with *C. jejuni*-associated GBS nor other host factors identified.

Phase variation of LOS structures has been shown in mucosal pathogens such as *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Haemophilus* species (22) and results in the heterogeneity of core oligosaccharide length, variation in sugar composition, and variability in linkages and sugar substitutions (27). The ability of *C. jejuni* to undergo phase variation in LOS biosynthesis genes has been demonstrated in vitro (7, 8, 10, 13). It is evident that one *C. jejuni* strain has the ability to produce a repertoire of LOS molecules, and this variability of cell glycolipids may provide an advantage during the course of *C. jejuni* infection. In fact, insertional inactivation of *cgtA* in *C. jejuni* 81-176, which results in loss of an *N*-acetylgalactosamine residue, thereby producing a truncated LOS core structure resembling GM_3 ganglioside, resulted in a significant increase in invasion of intestinal epithelial cells in vitro (10). Also, variation in sialylation of *C. jejuni* LOS affects the immunogenicity and serum resistance of *C. jejuni* (9). Hence, current data suggest that many strains of *C. jejuni* can synthesize ganglioside mimics (15, 21) and have the ability to convert between different LOS structures (7, 8, 10, 12, 13). Although this would appear to have serious consequences for the development of a successful vaccine, particularly using an attenuated approach, in our study the development of ganglioside mimicry in the reference strain did not necessarily lead to the induction of antiganglioside antibodies in the experimentally infected volunteers. The use of inactivated *C. jejuni* strains lacking evidence of ganglioside mimicry or subunits of *Campylobacter* not

associated with GBS pathogenesis are vaccine development concepts that circumvent the potential of in vivo phase variation concerns and potential GBS risk. On the other hand, an alternative would be to inactivate the genes involved in LOS core extension in *C. jejuni* 81-176 to yield a strain in which ganglioside mimicry is no longer expressed. Finally, in addition to the presence of ganglioside mimicry by *C. jejuni*, it is evident that other contributions of the host are necessary to trigger pathogenic antiganglioside antibody production, as suggested previously (21).

ACKNOWLEDGMENTS

The study protocol was approved by the Naval Medical Research Center Institutional Review Board and the U.S. Army Human Subjects Research Review Board (protocol # A7707) in compliance with all federal regulations governing the protection of human subjects.

Funding for this work was provided by U.S. Army Medical Materiel Development Activity to the U.S. Navy Work Unit number 643807A.849.D.A0002 and by the Irish Health Research Board (to A.P.M).

We thank H. Willison (Institute of Neurological Sciences, Glasgow, Scotland), P. Guerry (NMRC, Silver Spring, Md.), and B. Schwerer (Institute of Neurology, University of Vienna, Austria) for providing some of the antibody preparations used in this study, and Antex Biologics for providing *C. jejuni* 81-176 biomass for LOS extraction.

The opinions expressed in this paper are those of the authors and are not to be construed as reflecting the official policy of the Department of Navy, Department of Defense, or the U.S. Government.

REFERENCES

1. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infection in humans. J. Infect. Dis. **157:**472–479.
2. **Buzby, J. C., B. M. Allos, and T. Roberts.** 1997. The economic burden of *Campylobacter*-associated Guillain-Barré syndrome. J. Infect. Dis. **176** (Suppl. 2)**:**192–197.
3. **Dickinson, B. L., and J. D. Clements.** 1995. Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. Infect. Immun. **63:**1617–1623.
4. **Downs, A., and W. Pigman.** 1976. Qualitative and quantitative determination of sialic acids. Methods Carbohydr. Chem. **7:**233–240.
5. **Freudenberg, M. A., A. Fomsgaard, I. Mitov, and C. Galanos.** 1989. ELISA for antibodies to lipid A, lipopolysaccharides and other hydrophobic antigens. Infection **17:**322–328.
6. **Frost, J.** 2001. Current epidemiological issues in human campylobacteriosis. J. Appl. Microbiol. **90**(Suppl.)**:**85–95.
7. **Gilbert, M., J. R. Brisson, M. F. Karwaski, J. Michniewicz, A. M. Cunningham, Y. Wu, N. M. Young, and W. W. Wakarchuk.** 2000. Biosynthesis of ganglioside mimics in *Campylobacter jejuni* OH4384. J. Biol. Chem. **275:** 3896–3906.
8. **Gilbert, M., M. F. Karwaski, S. Bernatchez, N. M. Young, E. Taboada, J. Michniewicz, A. M. Cunningham, and W. W. Wakarchuk.** 2002. The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*: biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. J. Biol. Chem. **277:**327–337.
9. **Guerry, P., C. P. Ewing, T. E. Hickey, M. M. Prendergast, and A. P. Moran.** 2000. Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni.* Infect. Immun. **68:**6656–6662.
10. **Guerry, P., C. M. Szymanski, M. M. Prendergast, T. E. Hickey, C. P. Ewing, D. L. Pattarini, and A. P. Moran.** 2002. Phase variation of *Campylobacter jejuni* 81–176 lipooligosaccharide affects virulence. Infect. Immun. **70:**787–793.
11. **Korlath, J. A., M. T. Osterholm, L. A. Judy, J. C. Forfang, and R. A. Robinson.** 1985. A point source outbreak of campylobacteriosis associated with consumption of raw milk. J. Infect. Dis. **152:**592–596.
12. **Linton, D., A. V. Karlyshev, P. G. Hitchen, H. R. Morris, A. Dell, N. A. Gregson, and B. W. Wren.** 2000. Multiple *N*-acetylneuraminic acid synthetase (neuB) genes in *Campylobacter jejuni*: identification and characterization of the gene involved in sialylation of lipo-oligosaccharide. Mol. Microbiol. **35:**1120–1134.
13. **Linton, D., M. Gilbert, P. G. Hitchen, A. Dell, N. A. Gregson, and B. W. Wren.** 2000. Phase variation of a β-1,3-galactosyltransferase involved in generation of the ganglioside GM_1-like lipo-oligosaccharide of *Campylobacter jejuni.* Mol. Microbiol. **37:**501–514.
14. **Moran, A. P., and J. L. Penner.** 1999. Serotyping of *Campylobacter jejuni* based on heat-stable antigens: relevance, molecular basis and implications in pathogenesis. J. Appl. Microbiol. **86:**361–377.
15. **Moran, A. P., and M. M. Prendergast.** 2001. Molecular mimicry in *Campylobacter jejuni* and *Helicobacter pylori* lipopolysaccharides: contribution to gastrointestinal infections to autoimmunity. J. Autoimmun. **16:**241–256.
16. **Moran, A. P., E. T. Rietschel, T. U. Kosunen, and U. Zahringer.** 1991. Chemical characterization of *Campylobacter jejuni* lipopolysaccharides containing *N*-acetylneuraminic acid and 2,3-diamino-2,3-dideoxy-D-glucose. J. Bacteriol. **173:**618–626.
17. **Neisser, A., H. Bernheimer, T. Berger, A. P. Moran, and B. Schwerer.** 1997. Serum antibodies against gangliosides and *Campylobacter jejuni* lipopolysaccharides in Miller Fisher syndrome. Infect. Immun. **65:**4038–4042.
18. **Neisser, A., B. Schwerer, H. Bernheimer, and A. P. Moran.** 2000. Ganglioside-induced antiganglioside antibodies from a neuropathy patient cross-react with lipopolysaccharides of *Campylobacter jejuni* associated with Guillain-Barré syndrome. J. Neuroimunol. **102:**85–88.
19. **Prendergast, M. M., T. U. Kosunen, and A. P. Moran.** 2001. Development of an immunoassay for rapid detection of ganglioside GM_1 mimicry in *Campylobacter jejuni* strains. J. Clin. Microbiol. **39:**1494–1500.
20. **Prendergast, M. M., A. J. Lastovica, and A. P. Moran.** 1998. Lipopolysaccharides from *Campylobacter jejuni* O:41 strains associated with Guillain-Barré syndrome exhibit mimicry of GM_1 ganglioside. Infect. Immun. **66:** 3649–3655.
21. **Prendergast, M. M., and A. P. Moran.** 2000. Lipopolysaccharides in the development of the Guillain-Barré syndrome and Miller Fisher syndrome forms of acute inflammatory peripheral neuropathies. J. Endotoxin Res. **6:**341–359.
22. **Preston, A., R. E. Mandrell, B. W. Gibson, M. A. Apicella.** 1996. The lipooligosaccharides of pathogenic gram-negative bacteria. Crit. Rev. Microbiol. **22:**139–180.
23. **Saito, M., N. Kasai, and R. K. Yu.** 1985. In situ immunological determination of basic carbohydrate structures of gangliosides on thin-layer plates. Anal. Biochem. **148:**54–58.
24. **Schwerer, B., A. Neisser, R. J. Polt, H. Bernheimer, and A. P. Moran.** 1995. Antibody crossreactivities between gangliosides and lipopolysaccharides of *Campylobacter jejuni* serotypes associated with Guillain-Barré syndrome. J. Endotoxin Res. **2:**395–403.
25. **Scott, D. A.** 1997. Vaccines against *Campylobacter jejuni.* J. Infect. Dis. **176**(Suppl. 2)**:**183–188.
26. **Skirrow, M. B., and J. Benjamin.** 1980. Differentiation of enteropathogenic *Campylobacter.* J. Clin. Pathol. **33:**1122.
27. **Van Putten, J. P. M., and B. D. Robertson.** 1995. Molecular mechanisms and implications for infection of lipopolysaccharide variation in *Neisseria.* Mol. Microbiol. **16:**847–853.

Editor: J. N. Weiser